Advances in Brief

17-Allylamino-17-demethoxygeldanamycin Induces the Degradation of Androgen Receptor and HER-2/*neu* and Inhibits the Growth of Prostate Cancer Xenografts[1]

David B. Solit, Fuzhong F. Zheng,
Maria Drobnjak, Pamela N. Münster,
Brian Higgins,[2] David Verbel, Glenn Heller,
William Tong, Carlos Cordon-Cardo,
David B. Agus,[3] Howard I. Scher, and
Neal Rosen[4]

Program in Cell Biology [D. B. S., F. F. Z., P. N. M., N. R.], and Departments of Medicine [D. B. S., B. H., D. B. A., H. I. S., N. R.], Pathology [M. D., C. C-C.], and Epidemiology and Biostatistics [D. V., G. H.], Memorial Sloan-Kettering Cancer Center, New York, New York 10021

Abstract

Purpose: Ansamycin antibiotics, including 17-allylamino-17-demethoxygeldanamycin (17-AAG), inhibit Hsp90 function and cause the selective degradation of signaling proteins that require this chaperone for folding. Because mutations in the androgen receptor (AR) and activation of HER2 and Akt may account, in part, for prostate cancer progression after castration or treatment with antiandrogens, we sought to determine whether an inhibitor of Hsp90 function could degrade these Hsp90 client proteins and inhibit the growth of prostate cancer xenografts with an acceptable therapeutic index.

Experimental Design: The effect of 17-AAG on the expression of Hsp90 regulated signaling proteins in prostate cancer cells and xenografts was determined. The pharmacodynamics of target protein degradation was associated with the toxicology and antitumor activity of the drug.

Results: 17-AAG caused the degradation of HER2, Akt, and both mutant and wild-type AR and the retinoblastoma-dependent G_1 growth arrest of prostate cancer cells. At nontoxic doses, 17-AAG caused a dose-dependent decline in AR, HER2, and Akt expression in prostate cancer xenografts. This decline was rapid, with a 97% loss of HER2 and an 80% loss of AR expression at 4 h. 17-AAG treatment at doses sufficient to induce AR, HER2, and Akt degradation resulted in the dose-dependent inhibition of androgen-dependent and -independent prostate cancer xenograft growth without toxicity.

Conclusions: These data demonstrate that, at a tolerable dose, inhibition of Hsp90 function by 17-AAG results in a marked reduction in HER2, AR, and Akt expression and inhibition of prostate tumor growth in mice. These results suggest that this drug may represent a new strategy for the treatment of prostate cancer.

Introduction

Prostate cancer can be eradicated when localized, but systemic disease remains incurable. Androgen ablation is the standard treatment for advanced disease, but despite dramatic clinical responses, virtually all of the patients relapse (1). The mechanisms responsible for disease progression after castration or treatment with antiandrogens are complex and not fully understood. The AR[5] is expressed at normal or amplified levels in most patients with androgen-independent disease, and several gain of function mutations have been characterized (2–4). These include mutations within the ligand-binding domain that alter ligand-binding specificity and have been associated with clinical progression after antiandrogen therapy (4).

In a majority of cases, changes in the AR gene have not been identified suggesting that other mechanisms must be involved. Recent studies show that activation of receptor tyrosine kinase signaling pathways leads to phosphorylation of steroid receptors and their activation in a ligand-independent manner (5–8). In one experimental system, selection for prostate tumor cells that grow at low levels of androgen was associated with overexpression of the HER2 receptor tyrosine kinase (9). Thus, prostate cancer tumor growth after castration or treatment with hormone receptor antagonists may be mediated by AR mutation or its ligand-independent activation by upstream tyrosine kinase pathways.

Ansamycin antibiotics, exemplified by GM, are natural products that bind to a conserved pocket in the Hsp90 family of chaperone proteins (10–12). Hsp90 is not required for general cotranslational protein folding but does play a role in the refolding of proteins in cells exposed to stress (13, 14). It is also required for the conformational maturation of Raf and steroid receptors (15, 16). High concentrations of ansamycins prevent

[5] The abbreviations used are: AR, androgen receptor; GM, geldanamycin; 17-AAG, 17-allylamino-17-demethoxygeldanamycin; RB, retinoblastoma; PI3k, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; EPL, egg-phospholipid; PSA, prostate specific antigen.
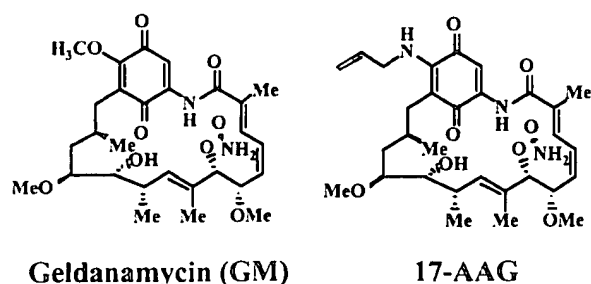
Geldanamycin (GM)   **17-AAG**

Fig. 1 Structures of GM and 17-AAG.

the binding of Hsp90 to its target proteins, which then cannot achieve their mature conformation (17). In addition, binding of ansamycins to the Hsp90-unfolded protein complex stabilizes the complex by preventing the ATP-dependent release of the chaperones (18, 19). The unfolded proteins in the complex are then ubiquinated and targeted for degradation in the proteasome (20, 21).

Occupancy of the Hsp90 pocket by GM causes the degradation of several signaling proteins important in mediating prostate cancer growth. These include AR and members of the HER family of receptor tyrosine kinases (15, 18, 20–22). Here we show that treatment of prostate cancer cell lines with the GM derivative 17-AAG (Fig. 1) results in the degradation of HER2, and both wild-type and mutant AR, growth arrest, and an RB-dependent G_1 block. Furthermore, at nontoxic doses, 17-AAG induces the degradation of AR and HER family tyrosine kinases in prostate tumors, and inhibits their growth. These data suggest that 17-AAG is effective in inhibiting pathways required for the growth of advanced prostate cancer. Moreover, ansamycins can inhibit Hsp90 function in mice without toxicity and, thus, may represent a new strategy for the treatment of this disease.

Materials and Methods

Materials. 17-AAG (NSC 330507) was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, National Cancer Institute (Bethesda, MD). Drug was dissolved in DMSO to yield 10 μM and 50 mg/ml stock solutions, and stored at −20°C. The following antibodies were used: Akt (Cell Signaling, Beverly, MA; 1:500), AR (PharMingen, San Diego, CA; 1:250 for immunoblot and 1:100 for immunofluorescence), HER2 (Santa Cruz Biotechnology, Santa Cruz, CA; C-18, 1:1000), HER3 (Santa Cruz Biotechnology; C-17, 1:1000), Hsp70 (StressGene, Victoria, British Columbia, Canada; 1:1000), Hsp90 (StressGene; 1:1000), MAPK (Cell Signaling; 1:1000), p85 subunit of PI3k (Upstate Biotechnology, Lake Placid, NY; 1:2000), and RB (PharMingen; 1:1000).

Cell Culture. The human prostate cancer cell lines LNCaP, DU-145, and PC-3 were obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 supplemented with 5–10% heat-inactivated fetal bovine serum, 2 mM glutamine, and 50 units/ml of penicillin and streptomycin in a humidified 5% CO_2/air atmosphere at 37°C.

LAPC-4 was generously provided by Charles Sawyers (UCLA, Los Angeles, CA) and maintained in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum and 10 nM R1881.

For the Alamar Blue proliferation assay, $2-4 \times 10^3$ cells were plated in 96-well plates. Later (48 h), cells were treated with 17-AAG for 96 h or 0.01% DMSO as control. On day 4, Alamar Blue viability assay (AccuMed, Westlake, OH) was performed as described elsewhere (23). IC_{50} and IC_{90}s were calculated as the doses of 17-AAG required to inhibit cell growth by 50 and 90%, respectively. Cell cycle distribution was assayed as described previously by Nusse *et al.* (24) with a Becton Dickinson fluorescence-activated cell sorter and analyzed by the Cell Cycle Multicycle system (Phoenix Flow System, San Diego, CA).

Immunoblotting. For immunoblotting, cells in culture were harvested in medium, washed twice in PBS, and then dissolved in SDS lysis buffer [50 mM Tris-HCl (pH 7.4) and 2% SDS], boiled for 10 min, and sonicated briefly. Cell lysates were cleared by centrifugation at $14,000 \times g$ for 10 min, and supernatants were collected as the experimental samples. Lysates were added to sample buffer [0.3125 M Tris-HCl (pH 6.8), 10% SDS, 50% glycerol, and 77.5 mg/ml DTT], and equal amounts of protein were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Blots were blocked in 5% nonfat milk in Tris-buffered saline [0.1% Tween 20, 10 mM Tris (pH 7.4), and 150 mM NaCl] and subsequently probed with the antibody of interest. After incubation with horseradish peroxidase-conjugated secondary antibodies, proteins were visualized by chemiluminescence (Amersham Corp., Piscataway, NJ). To prepare lysate from xenograft tumors, tumor tissue was homogenized in 2% SDS lysis buffer for 30 s then processed as above.

Immunofluorescence. For immunofluorescence, 5×10^3 cells were plated onto fibronectin-coated chamber slides (Fisher Scientific). Cells were then incubated with 17-AAG. 500 nM, or 0.01% DMSO (control). At the indicated time points, slides were washed twice with ice-cold PBS, and fixed with methanol and acetone solution (1:1) for 5 min. Fixed monolayers were rehydrated with water and then blocked with 3% BSA in PBS solution. After nonspecific blocking, cells were incubated with anti-AR monoclonal antibody in 1% BSA in PBS at room temperature then washed three times with PBS. Monolayers were then incubated with an Alexa-488 conjugated secondary antibody for 1 h at room temperature. Nuclei were stained with 0.5 μg/ml bis-benzimide (Hoechst 33342).

Animal Studies. Four- to six-week old *nu/nu* athymic male and female mice were obtained from the National Cancer Institute-Frederick Cancer Center (Frederick, MD) and maintained in ventilated caging. Experiments were carried out under an Institutional Animal Care and Use Committee-approved protocol, and institutional guidelines for the proper and humane use of animals in research were followed. Before administration, 17-AAG was dissolved in an EPL vehicle developed for this purpose by the National Cancer Institute. To aid in the identification of an optimal dose and schedule, nontumor bearing mice were treated by i.p. injection with 25–200 mg/kg of 17-AAG 5 days/week for 3 weeks or by the EPL vehicle alone. Serum samples were taken from each group, and equal volumes were pooled on days 5, 10, and 15 of treatment for serum
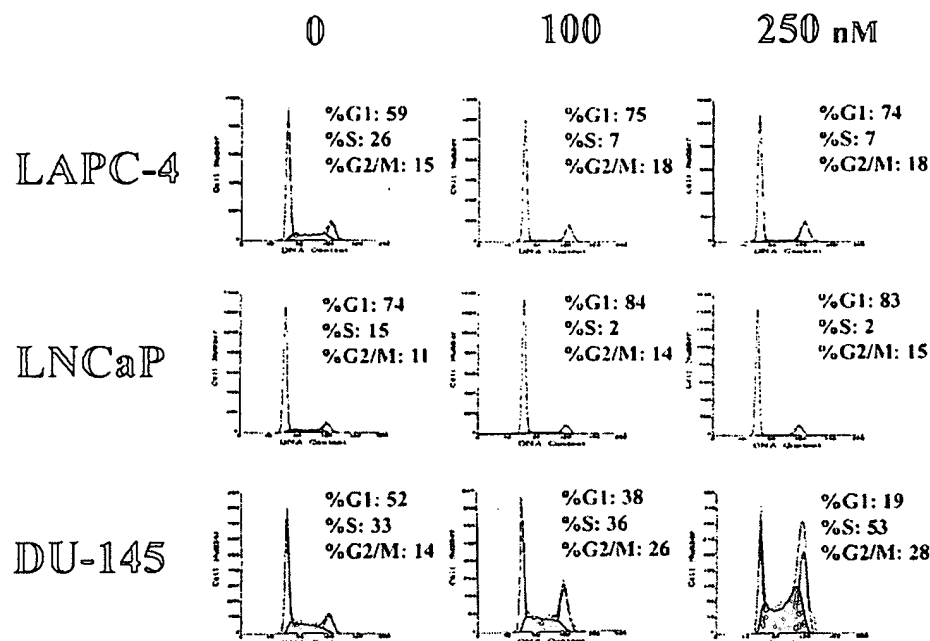
Fig. 2 17-AAG treatment of prostate cancer cell lines causes an RB-dependent cell cycle arrest. LAPC-4 (wild-type RB), LNCaP (wild-type RB), and DU-145 (mutant RB) cell lines were treated with 0 (0.01% DMSO), 100, or 250 nM 17-AAG for 48 h. Cell cycle distribution was assessed by fluorescence-activated cell sorter analysis. LAPC-4 and LNCaP cells arrested in G_1. By immunofluorescent staining, DU-145 cells arrested in mitosis (DNS).

chemistry and liver function analysis. At sacrifice, plasma samples were collected for complete blood count. A gross necropsy was performed on all of the mice, and a complete necropsy, including histopathology, was performed on 1 animal/group.

Male animals were inoculated s.c. with minced tumor tissue from donor mice bearing the androgen-dependent CWR22 xenograft line. Females were inoculated in the same way with the androgen-independent xenografts CWR22R and CWRSA6. These variants were derived from tumors that regrew after castration-induced regression of CWR22 tumors (25). Tumor cells were injected together with reconstituted basement membrane (Matrigel; Collaborative Research, Bedford, MA). To maintain stable serum testosterone levels, 12.5-mg 90-day sustained release testosterone pellets (Innovative Research of America, Sarasota, FL) were placed s.c. before inoculation with androgen-dependent tumor. Tumor dimensions were measured twice a week with vernier calipers, and tumor volumes were calculated with the formula: $\pi/6 \times$ larger diameter $\times$ (smaller diameter)^2. Mice with established tumors 4–5 mm in diameter were selected for study (n = 5–8 per treatment group). All of the mice received Augmentin (Amoxicillin/Clavulanate potassium; SmithKline Beecham) in their drinking water while on therapy. Mice were sacrificed by CO_2 euthanasia.

Both continuous and intermittent dosing schedules were studied. The "continuous" dosing schedule involved exposure to drug 5 days/week for 3 consecutive weeks. In the "intermittent" schedule, mice were treated with one 5-day cycle and then monitored for tumor progression. At progression, mice were treated with a second 5-day cycle of drug. In experiments with the androgen-dependent CWR22 tumor, serum PSA levels were measured with the PSA Assay kit (American Qualex Antibodies, San Clemente, CA).

In experiments designed to define the pharmacodynamic effects of 17-AAG on AR and HER-kinase expression, mice

with established tumors were treated with 17-AAG at the doses specified or with EPL alone. At the time of sacrifice, serum was collected, and tumors were flash frozen or fixed in 10% buffered formalin. Immunohistochemistry for AR (clone F39.4.1: 2 μg/ml; BioGenex, San Ramon, CA) and HER2 (HercepTest; DAKO Corp., Carpinteria, CA) were performed as described previously (25, 26). Serum concentrations of 17-AAG and 17-amino-17-demethoxygeldanamycin were determined by high-performance liquid chromatography based on the method of Egorin *et al.* (27).

Statistical Analysis. A permutation test was used to compare the average tumor volume over time between groups, using 5000 resamples. The null hypothesis of this test is that there is no difference in the change in tumor volumes over time between treatment groups. The statistic used to test this hypothesis was the sum of the squared differences between mean tumor volume summed over all time points, which in effect, compares the trajectories of the average tumor volume between treatment groups. It is defined as follows:

$$SS\_DEV = \sum_{i=1}^{k} (\bar{x}_i - \bar{y}_i)^2,$$

where there are k time points and $\bar{x}_i$ and $\bar{y}_i$ are the average tumor volumes at time i in each treatment group. The Wilcoxon sum-rank test was used to compare treatment and control group serum PSA values.

Results

17-AAG Inhibited Prostate Cancer Cell Proliferation by Causing an RB-dependent G_1 Growth Arrest. 17-AAG is a less toxic derivative of GM now in clinical trial. We found that

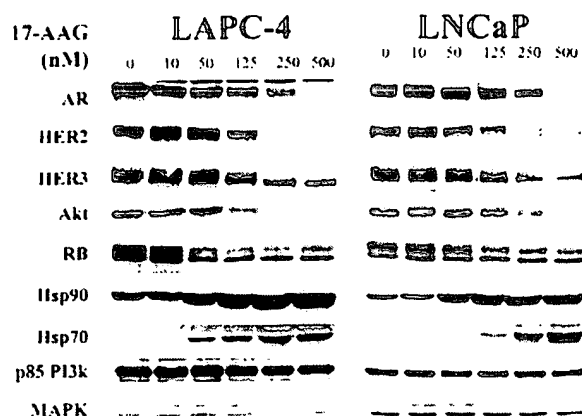| 17-AAG (nM)	LAPC-4						LNCaP					
	0	10	50	125	250	500	0	10	50	125	250	500
AR												
HER2												
HER3												
Akt												
RB												
Hsp90												
Hsp70												
p85 PI3k												
MAPK												

Fig. 3 Effect of 17-AAG on signaling proteins in LNCaP and LAPC-4 prostate cancer cells. Cells were treated for 24 h with 17-AAG at the indicated doses or DMSO (0.01%) as control. By immunoblot 17-AAG caused a dose-dependent decline in AR, HER2, HER3, and Akt expression and hypophosphorylation of RB. Levels of Hsp70 and Hsp90 were increased. No change in p85 PI3k or MAPK expression was noted.

17-AAG inhibited the anchorage-dependent growth of four representative prostate cancer cell lines. After 96 h of exposure, IC_{50}s ranged from 25–45 nM (LNCaP, 25 nM; LAPC-4, 40 nM; DU-145, 45 nM; and PC-3, 25 nM). At doses that caused complete growth arrest (75–125 nM for LNCaP and LAPC-4; and 150–200 nM for DU-145), prostate cell lines with intact RB (LNCaP, LAPC-4, and PC-3) arrested in G_1 after 17-AAG treatment, whereas DU-145, an androgen-independent cell line with mutated RB, arrested in mitosis (Fig. 2; data not shown). Growth arrest was accompanied by hypophosphorylation of RB (Fig. 3). These data are consistent with previous results in which we showed that the ansamycin Herbimycin A caused an RB-dependent G_1 block with RB-negative cells undergoing arrest in prometaphase (28, 29).

17-AAG Caused the Down-Regulation of HER2, HER3, and AR. Modulation of Hsp90 function by ansamycins causes the proteasomal degradation of a subset of cellular proteins. Most proteins and mRNAs are unchanged, as determined by evaluating multiple specific control proteins (PI3k and MAPK in Fig. 3 and DNS), the lack of change in the patterns of total cellular protein expression (DNS), and microarray analysis (30). HER2 is expressed at significant levels in both LAPC-4 and LNCaP, and is one of the most sensitive and rapidly degraded targets of 17-AAG (Fig. 3). HER3 and Akt levels also declined after 17-AAG treatment. 17-AAG increased the expression of the heat shock proteins Hsp90 and Hsp70.

Both wild-type AR (LAPC-4) and the Thr877Ala AR mutant found in LNCaP are sensitive to the drug as determined by immunoblot (Figs. 3 and 4). Exposure to 500 nM (24 h) of drug resulted in a 91% reduction in wild-type AR and a 92% reduction in the Thr877Ala AR mutant expressed by LNCaP cells. In LNCaP cells grown in serum-supplemented medium, most AR resides in the nucleus. Six and 12 h after 17-AAG treatment, nuclear AR staining was lost, and only faint cytoplasmic staining could be identified (Fig. 4). By 24 h, AR staining was again apparent with 52% of cells demonstrating detectable levels of nuclear AR by immunofluorescence. The intensity of nuclear AR staining within this population

was heterogeneous with only 10% of cell demonstrating nuclear AR staining equivalent to control levels (Fig. 4).

Toxicology Studies. The degradation of mutant AR and HER2 by 17-AAG suggests that this agent may be useful in the treatment of advanced prostate cancers. We sought to determine whether inhibition of these pathways could be accomplished *in vivo* with nontoxic doses of 17-AAG. The GM-Hsp90 domain has been studied in various species (yeast, bacteria, *Drosophila*, mouse, and human) and is highly conserved across species (11). Specifically, human and murine Hsp 90α have >99% homology. Therefore, we evaluated the toxicity profile and pharmacology of 17-AAG in tumor and nontumor bearing *nu/nu* athymic mice. 17-AAG is metabolized by hepatic microsomes into at least five metabolites (27). The major metabolite, 17-AG, is active and is equally potent in degrading HER2 and AR in LNCaP and LAPC-4 cell lines.[6] We measured 17-AAG and 17-AG plasma concentrations after i.p. administration of one 50 mg/kg dose. 17-AAG was rapidly absorbed, and peak levels of >10 μM were achieved within 30 min. Serum levels of 17-AG >1 μM were also detectable by high-performance liquid chromatography. Both 17-AAG and 17-AG were then rapidly cleared with no detectable plasma levels 8 h after injection.

We found that the maximally tolerated dose of 17-AAG was schedule dependent and higher in control mice than in tumor-bearing mice. In nontumor bearing mice, treatment with three consecutive 5-day cycles of 75 mg/kg or more caused toxicity as evidenced by weight loss, elevated liver transaminase levels, anemia, and death (1 of 4 mice at the 75 mg/kg dose level, 3 of 4 mice at 125 mg/kg, and 4 of 4 mice at 200 mg/kg). Necropsy of mice after treatment with 17-AAG or the vehicle alone revealed peritonitis, possibly related to the i.p. route of administration, but no other gross or histological abnormalities. With less frequent dosing, up to 150 mg/kg/day of drug could be safely administered without evidence of toxicity (weight loss or death).

17-AAG Caused a Reduction in AR, HER2, and HER3 Expression in Prostate Cancer Xenograft Tumors. To determine whether nontoxic doses of 17-AAG could induce down-regulation of AR and HER kinases *in vivo*, we studied the effects of 17-AAG on the expression of these cellular proteins in the CWR22 xenograft model. In CWR22, the AR contains a mutation (histidine->tyrosine at residue 874) located within the ligand-binding pocket (31). Despite this mutation, androgens are still required for the growth of this tumor. Castration of CWR22 tumor-bearing mice causes tumor regression followed 80–200 days later by a resumption of tumor growth (25). Several of these variants have been serially passaged, and two (CWR22R and CWRSA6) were selected for additional use.

We treated mice bearing CWR22 or CWRSA6 (androgen-independent) tumors with 25 or 50 mg/kg 17-AAG or EPL diluent for 4 days. After the final dose (8 h), the mice were sacrificed and the tumors removed. Four days of 17-AAG treatment resulted in a dose-dependent reduction in the expression of AR, HER2, HER3, and Akt (Fig. 5A; CWR22 DNS). A dose of 50 mg/kg resulted in an 87% decline in AR, a 85% decline in HER2, a 50% decline in HER3, and a 60% decline in Akt
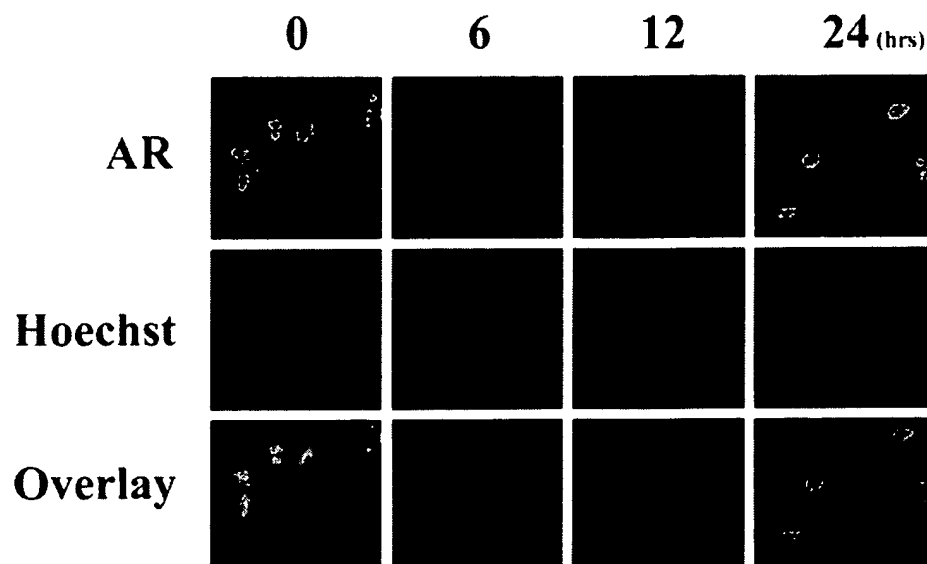
Fig. 4 Immunofluorescent staining of LNCaP cells for AR expression. Six and 12 h after treatment with 17-AAG (500 nM) LNCaP cells demonstrate a loss of nuclear AR expression. By 24 h a heterogeneous cell population is apparent with 52% of cells demonstrating at least partial recovery of AR staining.

expression in CWRSA6 tumors. Treatment was also associated with an 8-fold increase in Hsp70 and a 1.5-fold increase in Hsp90 levels. No change in the expression of PI3k was noted.

To characterize the kinetics of this effect, mice with well-established CWRSA6 tumors of comparable size were treated with a single dose of 17-AAG 50 mg/kg and sacrificed pretreatment and from 2 to 48 h afterward. A rapid, >50% decline in AR, HER2, and HER3 expression in the tumors was noted by 2 h (Fig. 5B). The maximal declines in AR and HER2 were noted at 4 h: a 97% reduction in HER2 and an 80% reduction in AR. By 24 h, AR expression returned to near baseline levels, whereas a rise in HER2 expression was not noted until 48 h after drug administration. The kinetics of HER2 recovery was similar in mice treated with 3 or 5 consecutive days of 17-AAG with its expression returning to near control levels 48 h after the final dose of therapy (DNS). With a single dose of therapy, the effect of 17-AAG on Akt expression was more delayed and less pronounced (maximum decline of 35% at 8 h) than that seen with AR, HER2, and HER3 (DNS).

In untreated CWRSA6 tumors, AR staining was compartmentalized in the nucleus (Fig. 5C). The frequency and intensity of this staining was diminished 4 and 8 h after 17-AAG administration. A steep decline in the mitotic index and a loss in membranous HER2 staining were also apparent at these time points. These data demonstrate that at nontoxic doses, 17-AAG induces the degradation of AR, HER2, HER3, and Akt in prostate tumors.

17-AAG Inhibited the Growth of Androgen-dependent and Androgen-independent Prostate Cancers. We studied the effects of two different dosing schedules of 17-AAG on the growth of CWR22, CWRSA6, and CWR22R tumors. The intermittent schedule consisted of a 5-day treatment cycle followed by a second cycle when definitive evidence of tumor regrowth occurred. The continuous schedule was comprised of three consecutive weekly 5-day cycles. Both regimens caused a dose-dependent delay in xenograft tumor growth in all three models (Table 1; Fig. 6). For example, with the continuous

schedule, 50 mg/kg 17-AAG caused 80% growth inhibition of CWRSA6 tumor growth when assessed on the day the controls required sacrifice [Table 1; Fig. 6A, mean tumor volume (treatment group) versus mean tumor volume (control) on day 29; $P < 0.01$]. With the intermittent schedule, 17-AAG caused 87% growth inhibition of CWRSA6 tumor growth (Table 1; Fig. 6B; $P < 0.01$). Similar results were noted with the parental CWR22 model and with a second androgen-independent subline CWR22R. Furthermore, in mice bearing CWR22 tumors, 17-AAG treatment (50 mg/kg dose level) was associated in an ~60% reduction in serum PSA (Day 25 serum PSA; intermittent schedule: 61% reduction versus control, $P < 0.01$; continuous schedule: 62% reduction, $P < 0.01$). These data demonstrate that at tolerable doses, 17-AAG inhibits the growth of prostate cancer cells in vivo. Additionally, growth inhibition correlates with a reduction in HER2, HER3, Akt, and the mutated AR (His874Tyr) expressed in the tumor.

Discussion

In this report, we examined the effects of 17-AAG on prostate cancer growth using a panel of prostate cell lines and the CWR22 xenograft model. 17-AAG is a less-toxic derivative of the ansamycin GM and is now in clinical trial. We found that 17-AAG causes the selective degradation of a subset of proteins, many of them involved in mitogenic signaling. These included both wild-type (LAPC-4) and mutant (LNCaP, CWR22) ARs, and the HER2 and HER3 receptor tyrosine kinases. The expression of Akt, which is downstream of HER2 and which may modulate AR signaling in tumors with HER2 overexpression, is also down-regulated by the drug (8). Most cellular proteins including MAPK and PI3k were unaffected.

We have shown previously that exposure of cancer cells to the ansamycins GM and herbimycin A leads to a loss of cyclin D-associated kinase activity and an RB-dependent G_1 growth arrest (28). Cells lacking RB-function progress through G_1
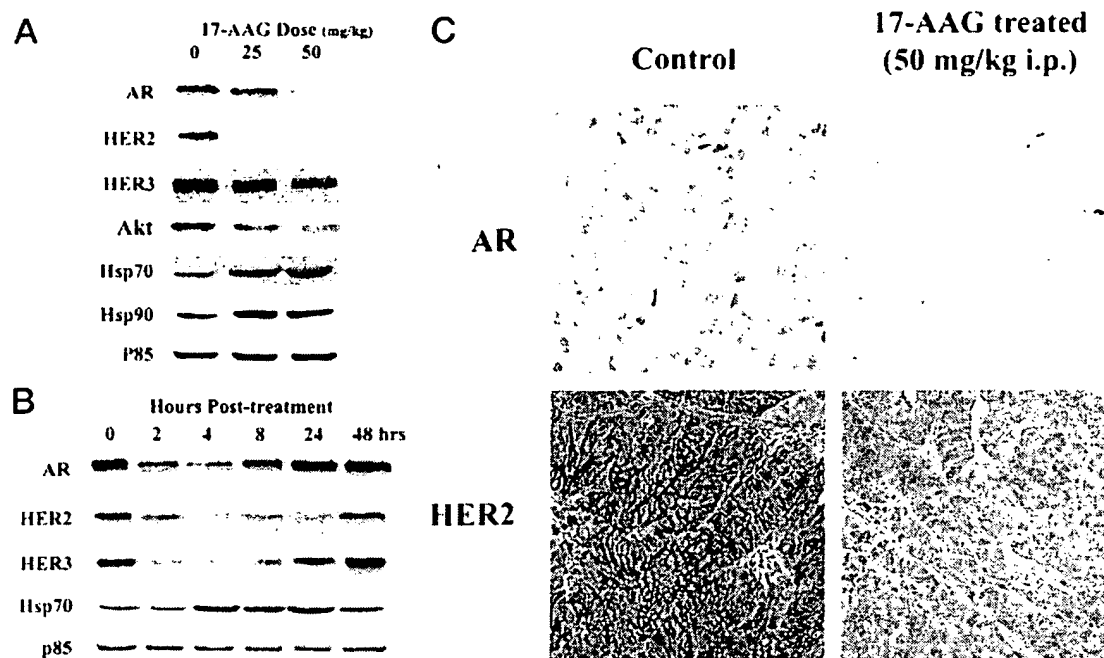
Fig. 5 *A*, changes in target protein expression of CWRSA6 xenograft tumors from mice treated with 17-AAG at doses of 25 mg/kg and 50 mg/kg daily for 4 days. Control mice received vehicle only. Mice were sacrificed 8 h after the final treatment on day 4. Immunoblot demonstrates a dose-de-·:·:dent decline in AR, HER2, HER3, and Akt expression. Levels of Hsp70 and Hsp90 were increased. No change in the control protein p85 PI3k wa: noted. This experiment was repeated with a second set of animals with equivalent results. *B*, mice with established CWRSA6 xenograft tumors tr. ite.! with one dose of 17-AAG 50 mg/kg. Pretreatment and at the time points specified, mice were sacrificed and tumors removed. By immunoblot, a rapid decline in HER2, HER3, and AR expression is evident within 2 h of treatment. No change in p85 PI3k expression occurred. This experiment was repeated with a second set of animals with equivalent results. *C*, immunohistochemistry of CWRSA6 xenograft tumors demonstrating a loss of nuclear AR staining, a loss in membranous HER2 staining, and a reduction in the mitotic index 4 h after treatment with one dose of 17-AAG 50 mg/kg. Similar findings were also noted 8 h after treatment.

Table 1 Effect of continuous and intermittent dosing schedules of 17:AAG on the growth of CWR22, CWR22R and CWRSA6 xenografts.

Tumor	Dose (mg/kg)	Dosing regimen	Percent (%) Inhibition (day)	P
CWR22	25	Continuous	42 (24)	<0.01
	50	Continuous	67 (24)	<0.01
	50	Intermittent	51 (25)	0.01
CWRSA6	25	Continuous	56 (29)	0.07
	50	Continuous	80 (29)	<0.01
	50	Intermittent	87 (30)	<0.01
CWR22R	25	Continuous	49 (21)	0.01
	50	Continuous	68 (21)	0.02
	50	Intermittent	67 (22)	<0.01

normally in the presence of drug and arrest in prometaphase before undergoing apoptosis. D-cyclins are not direct targets of ansamycins. but their expression is controlled at the post-transcriptional level by a PI3k/Akt kinase-dependent pathway (32). Consistent with these findings, LNCaP and LAPC-4 (wild-type RB) cells arrested in G_1, whereas DU-145, a cell line with mutant RB, arrested in mitosis.

We found that 17-AAG treatment reduced the expression of AR in prostate cancer xenografts and inhibited the growth of both androgen-dependent and -independent tumors. The maximum effect of 17-AAG on AR expression in the tumors was evident 4–8 h after treatment with receptor levels returning to baseline by 24 h. The degradation of wild-type and mutant AR by drug suggests that this class of agents may be particularly effective in the treatment of advanced androgen-independent prostate cancer. The mechanisms responsible for the emergence of androgen-independent disease are complex and not fully understood. In a subset of patients treated with AR antagonists, clinical progression is associated with AR gene amplification or mutation (4, 33). AR amplification may result in sufficient AR pathway activation to allow for tumor growth at low levels of testosterone. Mutations in AR may convert antagonists into agonists or lead to constitutive ligand-independent activation of the receptor. For example the missense mutation (Thr877Ala) found in LNCaP cells has been identified in patients treated previously with flutamide, and this AR exhibits altered ligand specificity (4). Hydroxyflutamide, adrenal androgens, and estrogens induce its activation and promote cell growth. In some patients, tumor regression may occur after flutamide withdrawal (4, 34). These data suggest that mutation or overexpression of AR may play a role in the progression to an androgen-independent state. Although the antiproliferative effects of ansamycins may be multifactorial and the result of inhibition of AR-independent pathways, the ability of ansamycins to degrade mutant as well as wild-type
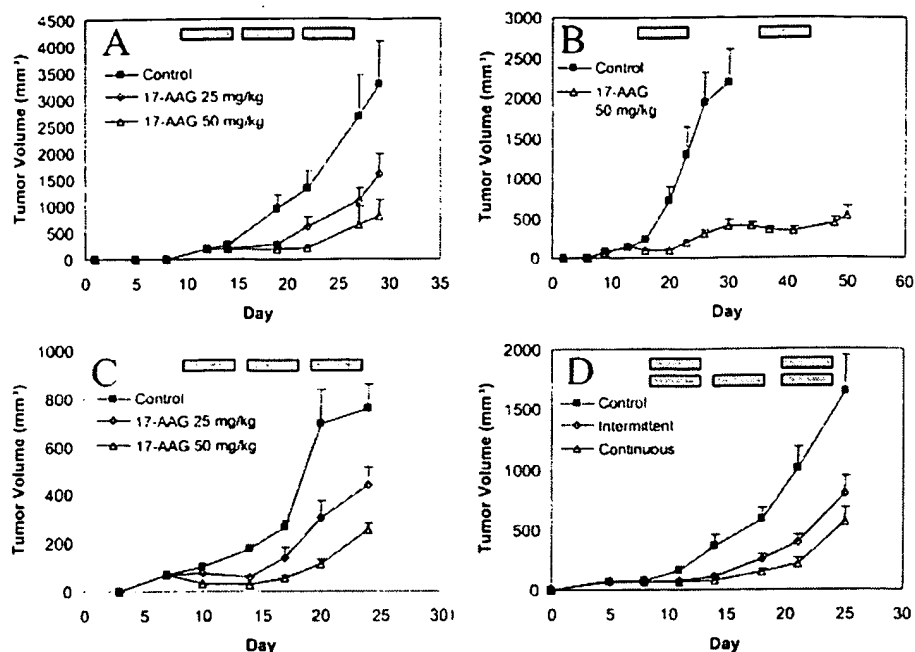
Fig. 6 A and B, response of CWRSA6 androgen-independent xenografts to 17-AAG. C and D, response of CWR22 androgen-dependent xenografts to 17-AAG. Bars, ± SE.

forms of AR suggest that they may be useful in the treatment of such patients.

In most patients whose tumors relapse after hormonal therapy, mutations or amplification of the AR gene have not been identified. Several recent studies suggest that modulation of AR activity by growth factor activated tyrosine kinase pathways allows for prostate cancer growth at low androgen levels. The epidermal growth factor, insulin-like growth factor-I, keratinocyte growth factor, and interleukin 6 all induce AR phosphorylation and the expression of PSA, a downstream target of AR (6, 35). Several lines of evidence suggest that HER2 may be important in this process. HER2 overexpression and gene amplification have been identified in a subset of patients with prostate cancer (36, 37). HER2 overexpression is more common in patients treated previously with hormonal therapy and those with androgen-independent metastatic disease (37). CWR22 and LNCaP both express high levels of HER2, and Herceptin (Trastuzumab), a humanized monoclonal antibody that binds to HER2, inhibits the growth of these xenografts (26). A mechanism for this effect has been proposed by Yeh et al. (7) who demonstrated that HER2 activates AR by inducing its phosphorylation by MAPK. As 17-AAG targets both the AR and HER2 for degradation and thus inactivation, tumors that depend on tyrosine kinase pathway-mediated phosphorylation of AR for growth and survival after androgen ablation may be particularly sensitive to this agent.

Akt, which is downstream of HER2, also phosphorylates AR and may modulate AR signaling in tumors with HER2 overexpression (8). We found that 17-AAG down-regulated the expression of Akt in a dose-dependent manner in the xenograft tumors, although this effect was delayed and of a lesser degree than the effect of 17-AAG on AR and HER2. Hsp90 has been reported recently to associate with Akt and regulate its activity (38). Therefore, Akt may be a direct target of 17-AAG. Alternatively, the reduction in

Akt expression caused by 17-AAG could be secondary to down-regulation of other pathways by the drug.

In this report, we show that 17-AAG, an inhibitor of Hsp90 function, delays the growth of prostate tumors in vivo at nontoxic doses. The inhibition of growth correlated with reduced expression of AR and HER-family kinases in the tumor. The results suggest that ansamycins may represent a new strategy for the treatment of advanced prostate cancers that depend on AR mutations or activated tyrosine kinases for tumor progression. Human trials to evaluate the efficacy of 17-AAG in this setting are now in progress.

Acknowledgments

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